RESEARCH ARTICLE

Bacterial community profiles in low microbial abundance sponges

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Abstract

It has long been recognized that sponges differ in the abundance of associated microorganisms, and they are therefore termed either 'low microbial abundance' (LMA) or 'high microbial abundance' (HMA) sponges. Many previous studies concentrated on the dense microbial communities in HMA sponges, whereas little is known about microorganisms in LMA sponges. Here, two LMA sponges from the Red Sea, two from the Caribbean and one from the South Pacific were investigated. With up to only five bacterial phyla per sponge, all LMA sponges showed lower phylum-level diversity than typical HMA sponges. Interestingly, each LMA sponge was dominated by a large clade within either *Cyanobacteria* or different classes of *Proteobacteria*. The overall similarity of bacterial communities among LMA sponges determined by operational taxonomic unit and UniFrac analysis was low. Also the number of sponge-specific clusters, which indicate bacteria specifically associated with sponges and which are numerous in HMA sponges, was low. A biogeographical or host-dependent distribution pattern was not observed. In conclusion, bacterial community profiles of LMA sponges are clearly different from profiles of HMA sponges and, remarkably, each LMA sponge seems to harbour its own unique bacterial community.

Introduction

The association of sponges and microbial communities has long been recognized, and extensive efforts have been made to identify these microorganisms and to characterize the association (Taylor *et al.*, 2007; Webster & Taylor, 2012). Overall, more than 7500 sponge-derived 16S rRNA gene sequences are now available in public databases and these sequences are affiliated with 17 formally described phyla and several more candidate phyla (Simister *et al.*, 2012). Sequences were obtained from many studies using clone library (Kennedy *et al.*, 2008; Turque *et al.*, 2008), isolation and cultivation (Zhang *et al.*, 2008; Sipkema *et al.*, 2011), and denaturing gradient gel electrophoresis (DGGE; Mohamed *et al.*, 2008; Anderson *et al.*, 2010) techniques. The application of next-generation sequencing technologies to sponges revealed the presence of even more formally described phyla and candidate divisions (Webster *et al.*, 2010; Lee *et al.*, 2011; Jackson *et al.*, 2012; Schmitt *et al.*, 2012b). Phyla found in many different sponges and represented by many different sequences include *Actinobacteria*, *Acidobacteria*, *Cyanobacteria*, *Chloroflexi*, *Proteobacteria* and the candidate phylum *Poribacteria*.

However, microbial communities can vary considerably among different sponges with respect to both microbial abundance and diversity. Microbial abundance is often estimated by visual inspection of the sponge mesohyl for bacterial cells using transmission electron microscopy (TEM). In an early study, Levi & Porte (1962) were the first to identify granular particles present in the sponge mesohyl as microbial cells. Subsequent microscopy studies systematically investigated sponges for the presence of bacteria, and it was soon realized that differences in

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microbial abundance exist among sponges (Reiswig, 1974; Vacelet & Donadey, 1977; Wilkinson, 1978). Sponges associated with many microorganisms were termed 'bacterial sponges' (vs. 'corneal sponges'; Bertrand & Vacelet, 1971) or 'bacteriosponges' (vs. 'nonsymbiont-harbouring 'normal' sponges'; Reiswig, 1981) and later 'high microbial abundance (HMA) sponges' (vs. 'low microbial abundance (LMA) sponges'; Hentschel *et al.*, 2003). In one of these early studies, the microbial concentration of the sponge *Aplysina fistularis* was found to be approximately 8×10^{10} cells per mL of tissue, comprising 20% of total specimen volume (Reiswig, 1981). This extraordinarily high number of microorganisms was later confirmed for other HMA sponges (in a range of 10^8–10^{10} bacteria per g or mL of tissue) and was contrasted with a microbial concentration of 10^5–10^6 bacteria per g or mL in LMA sponges (Hentschel *et al.*, 2006). The latter concentration is typical for natural seawater (Hentschel *et al.*, 2006).

LMA and HMA sponges not only differ in microbial abundances but also in microbial diversity. Weisz *et al.* (2007a) used TEM to identify two HMA and one LMA sponge from the Caribbean. Subsequent DGGE analysis revealed multiple bands in HMA sponges, and many of the respective 16S rRNA gene sequences were closely related to previously sponge-derived sequences. In contrast, DGGE from the LMA sponge resulted in few bands and the 16S rRNA gene sequence of the most dominant band showed highest affinity to seawater bacteria (Weisz *et al.*, 2007a). A clear difference in bacterial diversity was also found in a clone library study using the HMA sponge *Ancorina alata* and the LMA sponge *Polymastia* sp. (Kamke *et al.*, 2010). Analysis of a similar number of 16S rRNA gene sequences from both sponges revealed the presence of eight phyla in the HMA sponge but only two phyla in the LMA sponge. This trend was confirmed in a subsequent 454 pyrosequencing study where members of 15 phyla were detected in *A. alata* but members of only eight phyla in *Polymastia* sp. (Schmitt *et al.*, 2012b). The investigation of a single bacterial phylum in three HMA and three LMA sponges resulted in a similar pattern: *Chloroflexi* bacteria are more abundant and diverse in HMA than LMA sponges (Schmitt *et al.*, 2012a).

Further differences between LMA and HMA sponges that often co-occur in the same habitat have been described. For example, while the reproductive stages of HMA sponges generally contained large amounts of microorganisms, as evidenced by TEM, the reproductive stages of LMA sponges generally appeared bacteria-free (Schmitt *et al.*, 2007). Moreover, the sponge metabolic status seems to be influenced by microorganisms only in HMA and not in LMA sponges (Weisz *et al.*, 2007a; Ribes *et al.*, 2012). Microbial abundances might also have an influence on sponge morphology and physiology. A variety of HMA sponges had a denser mesohyl and a more complex aquiferous system with much slower pumping rates than LMA sponges (Weisz *et al.*, 2007b). Finally, LMA and HMA sponges also show differences in their chemistry. A recent study by Hochmuth *et al.* (2010) revealed that *supA* type polyketide synthase (PKS) genes were present in all six HMA sponges but were absent from six LMA sponges. HMA sponges also showed characteristic fatty acid profiles not found in LMA sponges (Hochmuth *et al.*, 2010).

Despite these previous results, there are still many unanswered questions regarding this sharp dichotomy between LMA and HMA sponges. This is also owing to the fact that many studies concentrated either on HMA sponges or did not distinguish between HMA and LMA sponges. In this study, we investigated bacterial communities in the tropical LMA sponges *Crella cyathophora* and *Stylissa carteri* from the Red Sea, in the tropical Caribbean sponges *Callyspongia vaginalis* and *Niphates digitalis*, and in the temperate sponge *Raspailia topsenti* from the South Pacific (New Zealand). We provide detailed data on the diversity and compare the bacterial community among LMA sponges and between bacterial communities in HMA sponges to elucidate the similarity of LMA sponge–microorganism associations.

Materials and methods

Sample collection

The sponges *C. cyathophora* and *S. carteri* were sampled in November 2010 by SCUBA at Fsar Reef in the Red Sea (22°23′N; 39°03′E) at a depth between 13 and 15 m. The sponges *C. vaginalis* and *N. digitalis* were collected in July 2003 off Little San Salvador Island, Caribbean Sea, Bahamas (24°34′N; 75°58′W), at a depth of 12 m. *Raspailia topsenti* was collected in February 2008 at Matheson's Bay on New Zealand's North Island (174°47′S, 36°18′E) at a depth of 3 m. After collection, sponge tissue was immediately washed with calcium–magnesium-free artificial seawater (CMF-ASW) 1–3 times and stored at − 80 °C until further use. Additionally, small sponge tissue pieces of *c.* 0.5 cm^3 from the Red Sea sponges were preserved in 2.5% glutaraldehyde-H_2O_{dd} for further processing by TEM.

Transmission electron microscopy

Fixed samples of Red Sea sponges were washed five times in cacodylate buffer (50 mM, pH 7.2), fixed in 2% osmium tetroxide for 90 min, washed again five times in

H_2O_{dd} and incubated overnight in 0.5% uranyl acetate. Following dehydration in an ethanol series (30%, 50%, 70%, 90%, 96% and three times in 100% for 30 min each), the samples were incubated three times for 30 min in 1 × propylene oxide, maintained overnight in 1 : 1 (vol/vol) propylene oxide-Epon 812 (Serva), incubated twice for 2 h in Epon 812 and finally embedded in Epon 812 for 48 h at 60 °C. Samples were then sectioned with an ultramicrotome (OM U3; C. Reichert, Austria) and examined by TEM (Zeiss EM 10; Zeiss, Germany).

DNA extraction and construction of 16S rRNA gene libraries

For the Caribbean and Red Sea sponges (one individual each), an initial cell lysis step was performed with 200–250 mg of tissue (frozen wet weight) from each sponge in 600 μL of 1% 2-mercaptoethanol RLT buffer (Qiagen, Germany) using a FastPrep® Instrument (MP Biomedicals). DNA was then extracted using the Allprep DNA/RNA mini Kit (Qiagen). DNA of *R. topsenti* was extracted from 5 to 6 mg of freeze-dried and ground tissue (separately from three individuals; PCR product was pooled afterwards) using a CTAB-based protocol as described previously (Taylor *et al.*, 2004). Briefly, cells were disrupted by bead-beating in an ammonium acetate extraction buffer containing chloroform/isoamyl alcohol (24 : 1). DNA was precipitated with 3 M sodium acetate and isopropanol, then washed in 70% ethanol, dried and redissolved in H_2O_{dd}. The quality and quantity of extracted DNA was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific).

A *c.* 1500-bp fragment of the 16S rRNA gene was amplified from all sponge samples using the primer pairs 27f/1492r (Lane, 1991) for the Caribbean and Red Sea sponges and 616V/1492r (Lane, 1991; Juretschko *et al.*, 1998) for *R. topsenti*. Cycling conditions were as follows: initial denaturing step at 96 °C for 4 min, followed by 35 cycles of denaturing at 96 °C for 60 s, primer annealing at 56 °C for 60 s and elongation at 72 °C for 90 s and a final extension step at 72 °C for 5 min. PCR products were cleaned using the QIAquick PCR Purification kit (Qiagen) and cloned either into the pCR® II-TOPO® vector (Invitrogen) or into the pGEM-T-easy vector (Promega). Clone libraries were constructed according to the manufacturer's instructions. Correct-sized inserts were identified by PCR using the vector-specific primer pairs M13f (5′ GTAAAACGACGGCCAG 3′) and M13r (5′ CAGGAAACAGCTATGAC 3′) or SP6 (5′ TAT TTA GGT GAC ACT ATA G 3′) and T7 (5′ TAA TAC GAC TCA CTA TAG GG 3′). PCR products were sequenced by the KAUST Genomics Core Lab on a 3730XL 96 Capillary Sequencing machine (Applied Biosystems) and at Macrogen (South Korea) with the PCR primers used for clone library construction. Poor-quality sequences as well as chimeric sequences detected with Pintail (Ashelford *et al.*, 2005) were removed from the data set. Final sequence data were submitted to the DDBJ/EMBL/GenBank databases under accession numbers JQ062599–JQ062861 and JN850797–JN850860.

Sequence analysis and phylogenetic tree construction

Operational taxonomic units (OTUs) and Chao1 values were calculated at a 98% sequence similarity threshold using Mothur v.1.22.2 (Schloss *et al.*, 2009). For comparison, 85 16S rRNA gene sequences obtained from the New Zealand LMA sponge *Polymastia* sp. (Kamke *et al.*, 2010) were also analysed. To determine the similarity of microbial communities in different environments, hierarchical cluster analysis was performed using an unweighted UniFrac algorithm (Lozupone *et al.*, 2006). 16S rRNA gene sequences from this study, as well as their closest relatives identified by initial BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.*, 1990), were automatically aligned to a SILVA reference alignment using the SINA Webaligner and merged into the SILVA version 108 database (Ludwig *et al.*, 2004; Pruesse *et al.*, 2007). The alignment was then manually refined using the editor tool in the ARB software package. The alignment is available upon request. Distance (neighbour-joining), maximum parsimony and maximum likelihood trees were calculated in ARB using long ($\geq$ 1200 bp) sequences only. Short sequences were added using the parsimony interactive tool in ARB without changing the tree topology. Phylogenetic consensus trees, using the maximum likelihood tree as a backbone, were manually constructed (Ludwig *et al.*, 1998). Maximum parsimony bootstraps (100 resamplings) were performed to further assess the stability of observed branching patterns.

Results

Sponge microbial abundance classification using TEM

To estimate the abundance of microorganisms in the mesohyl and to group the sponges into either LMA or HMA sponge categories, the mesohyl of three individuals of the Red Sea sponges *C. cyathophora* and *S. carteri* was inspected by TEM (5 fields and at least 2 sections per sponge). All investigated tissue sections were free of microbial cells (Fig. 1). Therefore, *C. cyathophora* and *S. carteri* are classified as LMA sponges. The Caribbean

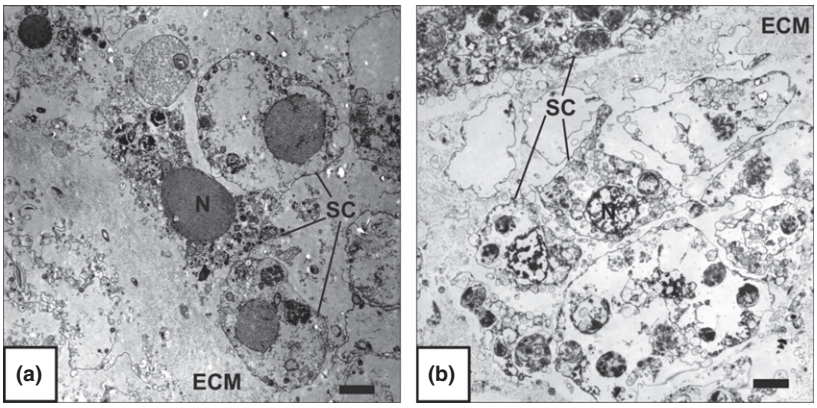

Fig. 1. TEM of the sponge mesohyl of *Crella cyathophora* (a) and *Stylissa carteri* (b). The mesohyl is free of bacterial cells. ECM, extracellular matrix; N, sponge cell nucleus; SC, sponge cell. Scale bar indicates 2 μm.

sponges *C. vaginalis* and *N. digitalis* as well as the New Zealand sponge *R. topsenti* were previously identified as LMA sponges because of the absence of large amounts of bacteria in the mesohyl (Schmitt *et al.*, 2007, 2012a).

Phylum level diversity

A total of 67, 67, 62, 67 and 9 nearly full-length 16S rRNA gene sequences were obtained from the sponges *C. cyathophora*, *S. carteri*, *C. vaginalis*, *N. digitalis* and *R. topsenti*, respectively. In addition, 55 partial 16S rRNA gene sequences were also obtained from *R. topsenti*. Sequences were affiliated with seven different bacterial phyla in total among all sponges, with three to five different phyla within a single sponge (Fig. 2). The phyla *Actinobacteria*, *Nitrospira*, *Planctomycetes* and *Spirochaetes* were represented by only very few sequences (up to four sequences) and were only discovered in a single sponge species each. *Bacteroidetes* were represented by only up to five sequences per sponge but were found in all sponges except *R. topsenti*. Sequences classified as *Cyanobacteria* and *Proteobacteria* were found in all five sponges. Of the detected four classes of *Proteobacteria*, *Alpha-* and *Gammaproteobacteria* were found in four sponges, whereas *Delta-* and *Betaproteobacteria* were found in three and two sponges, respectively. It is apparent that each bacterial community is dominated by a single phylum or proteobacterial class: *Cyanobacteria* in *C. vaginalis*, *Alphaproteobacteria* in *C. cyathophora* and *N. digitalis*, *Betaproteobacteria* in *R. topsenti* and *Gammaproteobacteria* in *S. carteri* (Fig. 2).

Analysis of OTUs within each sponge

Sequences within each sponge were grouped into OTUs with a 98% similarity threshold and 26 (Chao1 value: 78,5), 46 (157), 19 (124), 12 (24) and 18 (63,5) OTUs were obtained for the sponges *C. cyathophora*, *S. carteri*, *C. vaginalis*, *N. digitalis* and *R. topsenti*, respectively

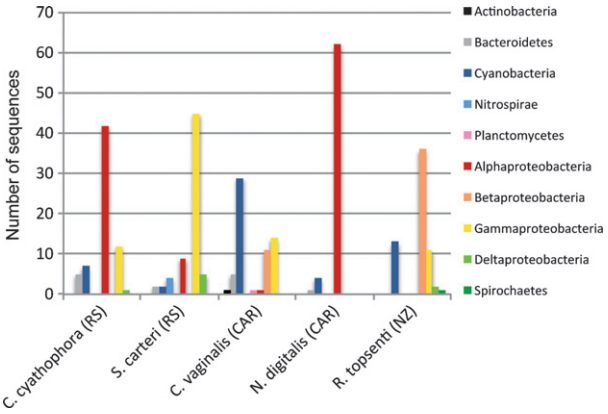

Fig. 2. Bacterial diversity on phylum level (and class level for *Proteobacteria*) within each of the five sponges. The number of sequences within each phylum or proteobacterial class is given. RS, Red Sea; CAR, Caribbean Sea; NZ, New Zealand.

(Fig. 3). All sponges contained mainly singleton OTUs that consisted of only one sequence (Fig. 3). Singleton OTUs comprised 75–81% of all OTUs. Singleton, doubleton (containing two sequences) and tripleton (containing three sequences) OTUs together comprised as much as 96% of all OTUs. Each sponge was dominated by one very large OTU. These biggest OTUs ranged from an OTU containing 10 sequences (15% of all sequences in *S. carteri*) to an OTU containing 54 sequences (81% of all sequences in *N. digitalis*). With the exception of the biggest OTU in *C. vaginalis,* which was affiliated with the *Cyanobacteria*, the biggest OTUs in all other sponges were affiliated with different classes of *Proteobacteria* (Fig. 3). For comparison, 85 16S rRNA gene sequences obtained from the New Zealand LMA sponge *Polymastia* sp. (Kamke *et al.*, 2010) were also analysed. Ninety-three percent of OTUs in *Polymastia* sp. were singletons and 96% were singletons and doubletons. The biggest OTU in *Polymastia* sp. contained 24 sequences (28% of all sequences in *Polymastia* sp.) and was affiliated with *Alphaproteobacteria*.

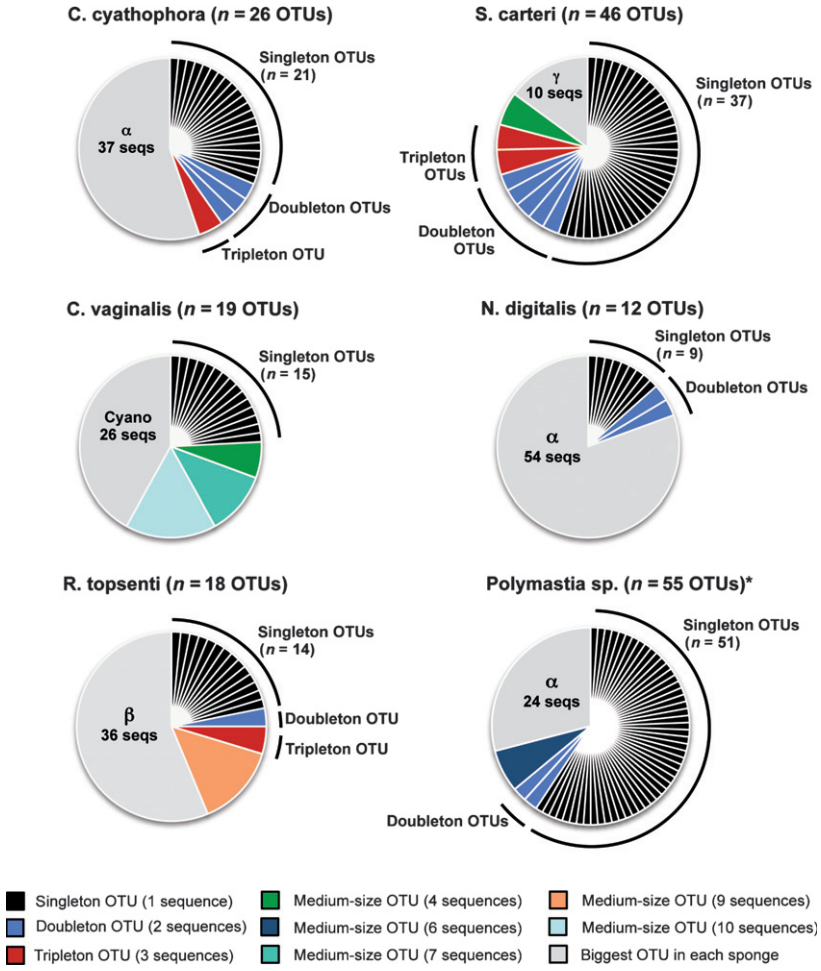

Fig. 3. 98% OTU analysis. The pie charts illustrate the number of OTUs per sponge, and the size of the respective sectors represents the number of sequences within each OTU. Singleton (consisting of one sequence), doubleton (two sequences) and tripleton (three sequences) OTUs are indicated. The biggest OTU (grey colour) in each sponge is highlighted and its size in terms of number of sequences and phylogenetic affiliation are given (α, *Alphaproteobacteria*; β, *Betaproteobacteria*; γ, *Gammaproteobacteria*, Cyano, *Cyanobacteria*). *For comparison, published sequence data from the New Zealand LMA sponge *Polymastia* sp. ($N = 85$) were also analysed (Kamke *et al.*, 2010).

Phylogenetic tree analysis

All 327 newly obtained 16S rRNA gene sequences were used together with appropriate reference sequences from sponges and other environments to construct phylogenetic trees (Fig. 4, Supporting Information, Fig. S1). A large clade containing 36 sequences from *R. topsenti* within the *Betaproteobacteria* corresponded to the largest OTU in this species (Fig. 3). Large clades were also found for *C. cyathophora*, *N. digitalis* and *S. carteri* within the *Alpha-* and *Gammaproteobacteria* (Fig. 4). Each of these clades contained several of the detected OTUs of the respective sponges. Whereas the clades of *N. digitalis* and *C. cyathophora* had no close similarity to other sponge-derived sequences, the clade of *S. carteri* showed some affinity to sequences derived from the sponge *Axinella corrugata* and the clade of *R. topsenti* fell within a sponge-specific cluster (SSC) (a monophyletic group of only sponge-derived sequences, see Hentschel *et al.*, 2002 for definition). Sequences from *C. vaginalis* were clustered in two smaller clades (11 and 10 sequences) within the *Beta-* and *Gammaproteobacteria*. The betaproteobacterial clade also formed a SSC together with other sponge-derived sequences (Fig. 4). The biggest OTU in *C. vaginalis* was affiliated with the *Cyanobacteria*. Phylogenetic tree analysis showed that this OTU fell within the *Synechococcus* lineage together with sequences from other sponges and environmental sequences (Fig. S1). In addition to the two already mentioned SSCs, two sponge-coral clusters (SCC) (SSCs that in addition contain sequences from corals, see Taylor *et al.*, 2007 for definition) were detected that were affiliated with *Gammaproteobacteria* and *Nitrospira* (Figs 4 and S1).

Similarity of bacterial communities among LMA sponges

When calculating 98% OTUs using 16S rRNA gene sequences from all sponges, 118 OTUs were obtained of which only two OTUs contained sequences from more than one sponge (Fig. 5a). The first comprised one sequence derived from *C. cyathophora* and a second sequence from *S. carteri*. This OTU was affiliated with the *Bacteroidetes*. The second OTU was bigger, with 28

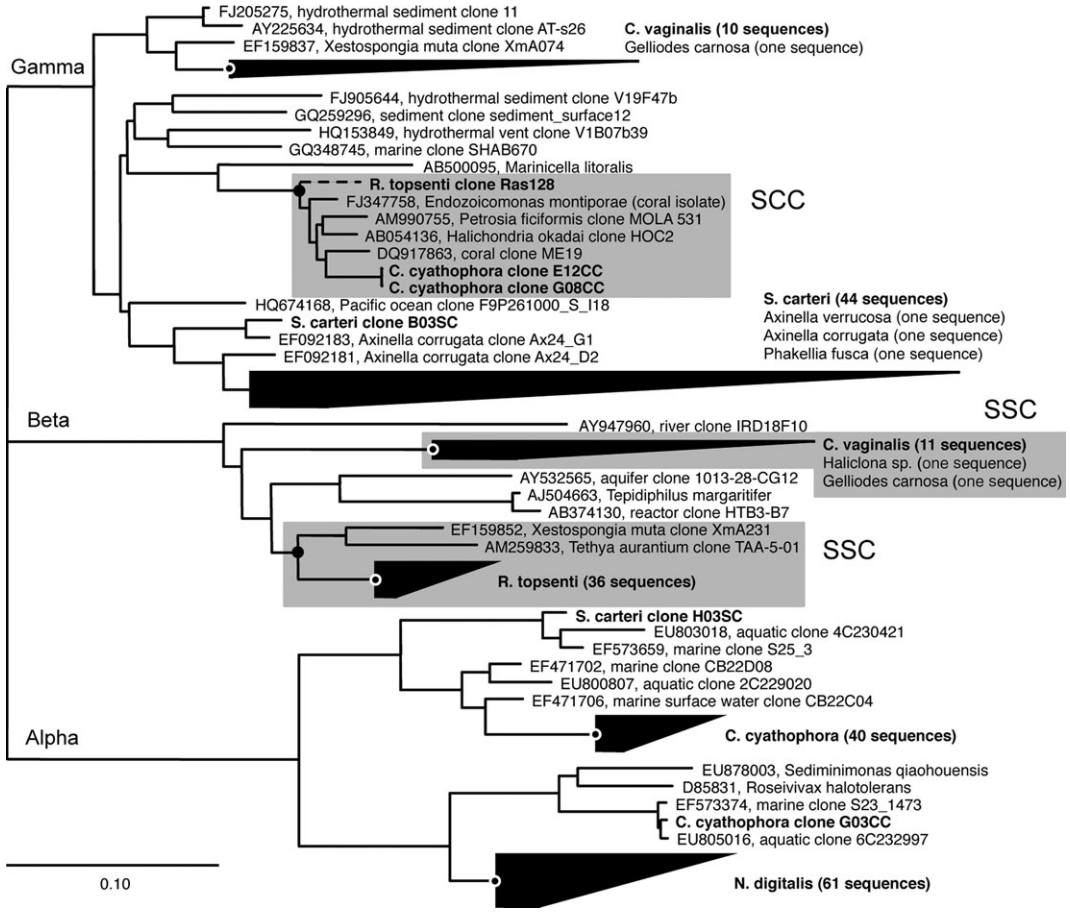

Fig. 4. 16S rRNA gene sequence-based maximum likelihood tree of *Proteobacteria*. Sponge-derived 16S rRNA gene sequences of this study are given in bold. The sponge names outside of wedges indicate species from which the respective sequences were derived. Numbers in parentheses indicate the number of sequences per sponge. Shaded boxes represent SSC and are either labelled as SSC or SCC depending on whether the respective cluster also contains a coral-derived sequence. Dashed line indicates a short sequence (< 1200 bp). Filled circles show bootstrap support of 100% for relevant groups. Scale bar indicates 10% sequence divergence.

sequences from both Red Sea sponges and both Caribbean species (Fig. 5a). It was affiliated with the *Cyanobacteria*.

Total bacterial communities were compared among all LMA sponges of this study using UniFrac analysis (Fig. 5b). For comparison, 85 sequences obtained from the New Zealand LMA sponge *Polymastia* sp. (Kamke *et al.*, 2010) were also included in the analysis. Most similar were the bacterial communities in the Caribbean sponge *N. digitalis* and the Red Sea sponge *C. cyathophora,* whereas the most distant communities were found in both New Zealand species. No obvious correlation with the sampling sites or with host phylogeny was detected (Fig. 5b).

Discussion

Microscopic techniques have long been used to estimate the abundance of bacteria in various sponges (Vacelet & Donadey, 1977; Wilkinson, 1978; Reiswig, 1981). Visual inspection of ultrathin sections of the Red Sea species *C. cyathophora* and *S. carteri* revealed the absence of bacteria in the mesohyl of both sponges (Fig. 1). Similarly, no bacterial cells were previously detected in the Caribbean sponges *C. vaginalis* and *N. digitalis* (Schmitt *et al.*, 2007). *Raspailia topsenti* was identified as LMA sponge because of the absence of large amounts of bacteria in the mesohyl (Schmitt *et al.*, 2012a). However, few bacteria-like structures were found in TEM micrographs, and similar structures were also occasionally detected in other LMA sponges such as *Mycale laxissima* (Schmitt *et al.*, 2007), *Niphates erecta* (Weisz *et al.*, 2007a), *Polymastia janeirensis* and *Hymeniacidon heliophila* (Turque *et al.*, 2008). It is possible that bacteria are generally missed in TEM surveys of LMA sponges because of their low abundances and/or because they only occur at certain sites within the sponge and are not equally distributed throughout the mesohyl as seen in HMA sponges (for

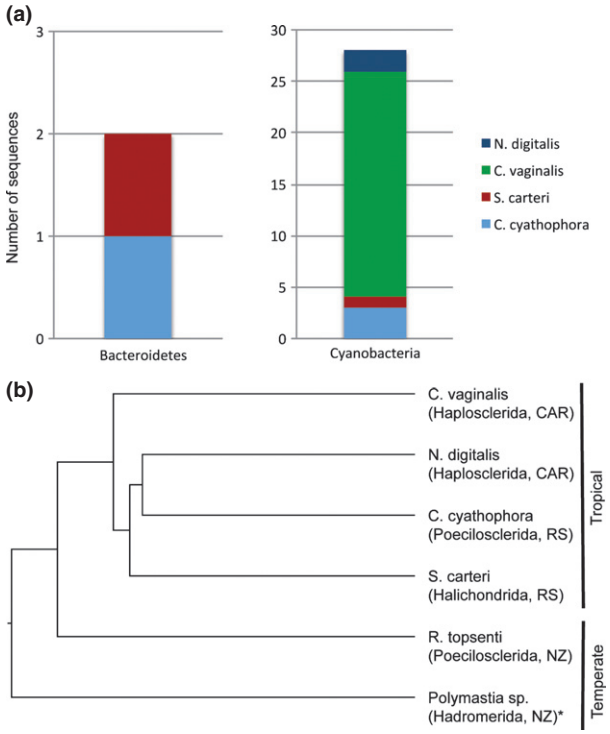

Fig. 5. Bacterial community similarity among LMA sponges. (a), 98% OTU analysis of all new sequences. The two 98% OTUs that contain sequences from different sponges are shown. (b), UniFrac cluster analysis of total bacterial communities. The taxonomic order and sample location of each sponge is given in parenthesis. *For comparison, published sequence data from the New Zealand LMA sponge *Polymastia* sp. ($N = 85$) were also analysed (Kamke *et al.*, 2010). RS, Red Sea; CAR, Caribbean Sea; NZ, New Zealand.

TEM of HMA sponges see e.g. Friedrich *et al.*, 1999; Thoms *et al.*, 2003). Generally, the detected microorganisms in LMA sponges are extracellular and show no signs of digestion, but it remains unclear whether they represent food bacteria, seawater contaminants or long-term sponge associates. However, the different numbers of bacterial cells in LMA sponges relative to the dense microbial communities in HMA sponges are obvious, even to the inexperienced eye.

As expected based on previous reports (Weisz *et al.*, 2007a; Kamke *et al.*, 2010), the diversity of bacterial communities in LMA sponges was lower in comparison with the diversity in HMA sponges. On phylum level, two phyla were found in the LMA sponge *Polymastia* sp., whereas eight phyla were detected in the co-occurring HMA sponge *A. alata* (Kamke *et al.*, 2010). In this study, analysis of a similar number of sequences revealed slightly more (3–5) bacterial phyla in the LMA sponges, but many of these phyla are restricted to a single sponge and are represented by only very few sequences (Fig. 2). Only the phylum *Proteobacteria* was represented by many sequences in all investigated sponges and it was also the dominant phylum in other LMA sponges, for example, *Polymastia* sp. (Kamke *et al.*, 2010) and *H. heliophila* (Erwin *et al.*, 2011). Other typical phyla often found in sponges (Simister *et al.*, 2012) are missing in this study, for example, *Acidobacteria*, *Chloroflexi* and *Gemmatimonadetes*. Representatives of these phyla might therefore be indicative of HMA sponges. Overall, the bacterial community pattern in LMA sponges is characterized by a low phylum-level diversity with usually *Proteobacteria* as the most prominent phylum and the absence of other phyla typical of HMA sponges.

Notable is a single large OTU in the bacterial community of each sponge (Fig. 3) and correspondingly a large clade in the phylogenetic tree (Figs 4 and S1). The biggest OTU in *C. vaginalis* is affiliated with the cyanobacterial *Synechococcus* lineage that was also found in the other Red Sea and Caribbean sponges of this study (Figs 5a and S1). *Synechococcus*-like sequences were recovered from multiple sponges and many of these sequences comprise a large SSC indicating a widespread sponge associate tentatively termed '*Synechococcus spongiarum*' (Usher *et al.*, 2004; Steindler *et al.*, 2005; Erwin & Thacker, 2008). However, sequences obtained in this study did not fall into this sponge-specific clade but instead cluster with environmental sequences (Fig. S1). *Synechococcus* is a very abundant member of the bacterioplankton (Scanlan & West, 2002), and we might have detected a marine free-living form in the LMA sponges.

The largest OTU found in each of the other LMA sponges is affiliated with the *Proteobacteria*. Interestingly, each sponge harbours a different clade within the *Alpha-*, *Beta-* or *Gammaproteobacteria* (Fig. 4). Similarly, dominant proteobacterial phylotypes were also detected in clone library data of other LMA sponges such as *Haliclona* sp. (Sipkema *et al.*, 2009), *H. heliophila* (Erwin *et al.*, 2011), *Ianthella basta* (Luter *et al.*, 2010) and *Polymastia* sp. (Kamke *et al.*, 2010). The dominance of a proteobacterial group seems to be another characteristic of bacterial communities in LMA sponges; however, the underlying reasons remain subject to speculation. It was shown that vertical transmission, as a mechanism to obtain bacteria, seems to occur mainly in HMA sponges (Schmitt *et al.*, 2007). This indicates that LMA sponges may acquire their bacteria mainly from the environment (e.g. seawater, sediment). If these bacteria are taken up by a selective mechanism, then all individuals of a species should be dominated by the same proteobacterium. This seems to be the case in a DGGE analysis of the LMA sponge *N. digitalis* where three individuals showed identical DGGE banding patterns and the respective sequences showed the same closest match in a BLAST search (Weisz *et al.*, 2007a). Alternatively, it is also possible that the

largest OTU represents the bacterium that first populated the growing animal. In this case, inoculation occurs by chance and different bacteria may dominate different sponge individuals of the same species. Here, we cannot test this hypothesis because no distinction between individuals was made but this will be an interesting aspect for future research.

In this study, several approaches were used to determine the similarity of bacterial communities among LMA sponges. Both, OTU (Fig. 5a) and UniFrac (Fig. 5b) analyses indicate a small overlap. From 118 OTUs (obtained by analysis of all new sequences in this study), only two contain sequences from more than one sponge (Fig. 5a). The larger OTU within the *Cyanobacteria* is affiliated with the *Synechococcus* lineage (Fig. S1) and might represent a marine free-living form (see discussion above). The second OTU contains only two sequences in total from two sponges and is affiliated with the *Bacteroidetes* (Fig. 5a). These two sequences are very similar to other marine, aquatic and planktonic sequences (Fig. S1) and might also represent a potentially abundant and widespread marine bacterium.

UniFrac analysis was used to compare the entire microbial communities among LMA sponges. Neither a biogeographical pattern according to host sponge distribution (e.g. sponges collected from the same location harbour more similar bacterial communities) nor a distribution according to host phylogeny (e.g. closely related sponges harbour more similar bacterial communities) was observed (Fig. 5b). However, it is interesting that the bacterial communities in the two New Zealand species were most distant. These sponges are the only temperate specimens collected, whereas all other sponges are tropical animals. A similar trend was observed in a recent 454 pyrosequencing study using 32 sponges from eight tropical and temperate locations with most similar bacterial communities (on phylum level) in tropical sponges (Schmitt *et al.*, 2012b). This suggests that environmental factors such as temperature, salinity or nutrient levels might indeed influence the composition of microbial communities in sponges. A combination of environmental factors (shaded vs. nonshaded habitats) and host-related factors (vertical transmission of symbionts) were suggested to structure the complex microbial communities in three HMA *Ircinia* sponges (Erwin *et al.*, 2012). Different environmental and/or host-related factors might also be responsible for the different bacterial community patterns in LMA and HMA sponges.

Another phenomenon found in sponge microbiology is SSCs, that is, monophyletic clusters of only sponge-derived sequences (Hentschel *et al.*, 2002; Taylor *et al.*, 2007; Simister *et al.*, 2012). The number of SSC and SCC detected in this study was relatively low ($N = 4$; Figs 4 and S1). This is in agreement with previous observations that HMA sponges usually contain many of these clusters but only few are found in LMA sponges (Kamke *et al.*, 2010; Erwin *et al.*, 2011; Schmitt *et al.*, 2012a). It was recently speculated that there might be LMA SSCs (e.g. SSCs that only contain LMA sponge-derived sequences; Schmitt *et al.*, 2012a). This could not be confirmed in this study where three of the four detected SSCs probably contain sequences from both LMA and HMA sponges (Figs 4 and S1). The fact that two SSCs also contain sequences from corals might indicate a wider host-associated distribution of these bacteria than only in sponges. However, this is not a phenomenon typical for LMA sponges but is also well known from HMA sponges although the ecological implications are unclear (Simister *et al.*, 2012). A similarity to other invertebrate-associated bacteria, such as symbionts of ascidians, was not found. Therefore, each LMA sponge seems to harbour a unique bacterial community that is different from other LMA as well as from HMA sponges and also from other marine invertebrates.

In summary, our investigation of bacterial communities in LMA sponges revealed a pattern that is clearly different from bacterial associations with HMA sponges. A low abundance of microorganisms corresponded with a low phylum-level diversity. The community composition of each sponge was dominated by a single clade within either *Cyanobacteria* or different classes of *Proteobacteria*. Typical bacterial phyla of HMA sponges are missing in LMA sponges. Bacterial community similarity between LMA and HMA sponges was very low and might indicate that different environmental- or host-related factors shape bacterial communities in LMA and HMA sponges.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. 16S rRNA gene sequence-based maximum likelihood tree.

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